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Glycerol metabolism in the methylotrophic yeast *Hansenula polymorpha*: phosphorylation as the initial step

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Abstract. In *Hansenula polymorpha* glycerol is metabolized via glycerol kinase and NAD(P)-independent glycerol-3-phosphate (G3P) dehydrogenase, enzymes which hitherto were reported to be absent in this methylotrophic yeast. Activity of glycerol kinase was readily detectable when cell-free extracts were incubated at pH 7–8 with glycerol/ATP/Mg^{2+} and a discontinuous assay for G3P formation was used. This glycerol kinase activity could be separated from dihydroxyacetone (DHA) kinase activity by ion exchange chromatography. Glycerol kinase showed relatively low affinities for glycerol (apparent K_m = 1.0 mM) and ATP (apparent K_m = 0.5 mM) and was not active with other substrates tested. No inhibition by fructose-1,6-bisphosphate (FBP) was observed. Both NAD-dependent and NAD(P)-independent G3P dehydrogenases were present. The latter enzyme could be assayed with PMS/MTT and cosedimented with the mitochondrial fraction. Glucose partly repressed synthesis of glycerol kinase and NAD(P)-independent G3P dehydrogenase, but compared to several other non-repressing carbon sources no clear induction of these enzymes by glycerol was apparent. Amongst glycerol-negative mutants of *H. polymorpha* strain 17B (a DHA kinase-negative mutant), strains blocked in either glycerol kinase or membrane-bound G3P dehydrogenase were identified. Crosses between representatives of the latter mutants and wild type resulted in the isolation of, amongst others, segregants which had regained DHA kinase but were still blocked in the membrane-bound G3P dehydrogenase. These strains, employing the oxidative pathway, were only able to grow very slowly in glycerol mineral medium.

Key words: *Hansenula polymorpha* – Glycerol – Glycerol kinase – Dihydroxyacetone – Dihydroxyacetone kinase – Methanol – Methylotrophy – Regulation

Under aerobic conditions glycerol is usually first converted by the enzyme glycerol kinase into glycerol-3-phosphate (G3P), which is then oxidized to dihydroxyacetone phosphate (DHAP) by a membrane-bound G3P dehydrogenase, both in bacteria (Lin 1976) and yeasts (Gancedo et al. 1968; Sprague and Cronan 1977; Adler et al. 1985). Methylotrophic yeasts were considered an important exception: In these organisms, and in a few other yeast species (*Candida valida* and *Schizosaccharomyces pombe*), glycerol kinase was found to be lacking. Instead, enzymic evidence was obtained to suggest that glycerol was first oxidized to dihydroxyacetone (DHA) by an NAD-dependent glycerol dehydrogenase and then phosphorylated to DHAP by DHA kinase (May and Sloan 1981; May et al. 1982; Babel and Hofmann 1982). This oxidative pathway has become firmly established meanwhile for *S. pombe* by the isolation and characterization of glycerol-negative mutants, blocked in glycerol dehydrogenase or DHA kinase (Gancedo et al. 1986).

In methylotrophic yeasts, however, the situation turned out to be more complex. On the basis of enzyme (Babel and Hofmann 1982; Tani and Yamada 1987) and mutant studies (O'Connor and Quayle 1979; Kato et al. 1986) it has been concluded that in these organisms DHA kinase is not only involved in the metabolism of methanol and DHA (van Dijken et al. 1978) but also in that of glycerol. We recently (de Koning et al. 1987) reported the isolation of a methanol- and DHA-negative mutant of the methylotrophic yeast *Hansenula polymorpha* (strain 17B) blocked in DHA kinase but still able to grow at the normal rate with glycerol as the sole carbon and energy source. During growth of strain 17B on glycerol, DHA kinase activity remained undetectable. In further studies we initially were also unable to detect activities of glycerol kinase in both wild type *H. polymorpha* and in strain 17B.

In this paper we present evidence that in *H. polymorpha* glycerol is metabolized via the phosphorylative pathway, involving a glycerol kinase unusual both in kinetic properties and in its sensitivity to inhibition by hydrazine. The strongest evidence for the in vivo functioning of this pathway was obtained by the isolation and characterization of glycerol-negative mutants of strain 17B blocked in glycerol kinase or G3P dehydrogenase. A preliminary report of part of this work has been published elsewhere (de Koning and Dijkhuizen 1986).

Materials and methods

Microorganisms and cultivation

The procedures followed for the cultivation, maintenance, genetic crossing, mutagenesis and induction of enzymes of *Hansenula polymorpha* de Morais et Maya CBS 4732 wild type and mutants have been described previously (de Koning et al. 1987).

Abbreviations. DHA, dihydroxyacetone; G3P, glycerol-3-phosphate; EMS, ethyl methanesulphonate; MTT; 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyl tetrazolium bromide; PMS, phenazine methosulphate; FBP, fructose-1,6-bisphosphate

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Pichia pastoris CBS 704, *Pichia pinus* CBS 744, *Candida boidinii* 5777 p29, *Candida methylica* CBS 8030 (methylotrophic yeasts), *Pichia anomala* CBS 5759, *Kluyveromyces marxianus* CBS 712, *Wickerhamiella domercqii* CBS 4351 and *Rhodutorula graminis* CBS 2826 (non-methylotrophic yeasts) were maintained on YEPD agar at 4° C. Cultivation was at 30° C on the same medium as used for *H. polymorpha*, except that vitamins were added according to van der Walt and Yarrow (1984).

Extract preparation and enzyme assays

The procedures used for harvesting and disruption of cells have been described previously (de Koning et al. 1987). Large cell debris was removed by centrifugation at 2,500 × *g* for 5 min at 4° C and part of the supernatant (low spin extract) was used for the assay of NAD(P)-independent G3P dehydrogenase. The remainder of the supernatant was centrifuged at 40,000 × *g* for 30 min at 4° C. Low molecular weight compounds were removed by passage through a Sephadex G25 (fine) column (1 × 5 cm) as described by de Koning et al. (1987). This extract was used for all other enzyme assays. Enzyme assays were carried out at 37° C for *H. polymorpha* and at 30° C for all other yeasts. NAD(P)-independent G3P dehydrogenase (EC 1.1.99.5) was measured in low spin extracts using a modification of the assay described by Adler et al. (1985). The reaction mixture contained 12.5 mM Pipes/KOH, pH 7.2, 0.05 mM PMS, 0.5 mM MTT and 10 mM L-G3P. An absorption coefficient for reduced MTT at 550 nm of 8.1 mM^{-1} cm^{-1} was used to calculate the enzyme activity. Glycerol dehydratase (EC 4.2.1.30) was measured discontinuously, using the assay conditions of Schütz and Radler (1984). The 3-hydroxypropionaldehyde formation was measured at 355 nm using the assay described by Avigad (1983). Glycerol oxidase activity was measured polarographically in a Biological Oxygen Monitor with a Clark-type oxygen electrode. The assay mixture (4 ml) contained 100 mM potassium phosphate buffer pH 7.5; catalase, 20 µg, and 20 mM glycerol. The assays for DHA reductase (NAD-dependent glycerol dehydrogenase; EC 1.1.1.6) and DHA kinase (EC 2.7.1.28) have been described previously (de Koning et al. 1987). DHAP reductase (NAD-dependent G3P dehydrogenase; EC 1.1.1.94) was assayed spectrophotometrically at 340 nm in 50 mM glycylglycine buffer pH 7.6, containing 2 mM DHAP and 0.15 mM NADH. Glycerol kinase (EC 2.7.1.30) activity was measured by three different assays. (1) The continuous assay for G3P formation described by Wieland and Suyter (1957). The reaction mixture contained glycine (200 mM)-hydrazine hydrate (1 M) pH 9.8, 2 mM $MgCl_2$, 2 mM ATP, 2.5 mM glycerol, 0.4 mM NAD and 7 units of G3P dehydrogenase. (2) A continuous assay for glycerol-dependent formation of ADP. The assay mixture contained 50 mM Pipes/KOH pH 7.2, 1 mM phosphoenolpyruvate, 0.15 mM NADH, 4 mM ATP, 5 mM $MgCl_2$, 2.0 units of pyruvate kinase and 5 units of lactate dehydrogenase. After 3 min incubation to determine the endogenous activity, the reaction was started by the addition of 20 mM glycerol. The decrease of the NADH concentration in time was followed at 340 nm. (3) A discontinuous assay for the formation of G3P. The assay mixture (0.5 ml) contained 50 mM Pipes/KOH; pH 7.2, 5 mM $MgCl_2$, 5 mM ATP and 20 mM glycerol. The reaction was started by the addition of cell-free extract and after 10 min incubation stopped with 10 µl 10 M perchloric acid. After 10 min on ice, precipitated protein was removed by centrifugation and the supernatant carefully neutralized with 10 M KOH. After 20 min incubation at −5° C the precipitated $KClO_4$ was removed by centrifugation and the supernatant used to assay the G3P produced, essentially as described by Wieland (1974). The reaction mixture (1.0 ml) contained glycine (100 mM)-hydrazine hydrate (500 mM), 0.5 mM NAD and extract with up to 75 nmol G3P. The final pH of the reaction mixture was 9.5. The change in absorbance at 340 mM after the addition of G3P dehydrogenase (7 units) was followed in a Hitachi 124 spectrophotometer. An absorption coefficient for reduced NAD of 6.22 mM^{-1} cm^{-1} was used to calculate the G3P concentration in the sample. The rate of G3P formation was linear with incubation time and the amount of cell-free extract added. Formation of G3P was not observed when either glycerol, ATP of Mg^{2+} was omitted from the assay mixture.

DEAE-polyol chromatography

Ion exchange chromatography with a high performance liquid chromatography system was performed as described by Kremer and Hansen (1987), except that a potassium chloride gradient was used.

Biochemicals and enzymes

Triosephosphate isomerase, glycerol kinase, G3P dehydrogenase and glycerol dehydrogenase were from Boehringer, Mannheim, FRG. Xylulose-5-phosphate was purchased from Sigma Chemical Co., St. Louis, MO, USA. Hydrazine hydrate (24%, suprapur) was a product from Merck, Darmstadt, FRG, and ethyl methanesulphonate from BDH Chemicals Ltd, Poole, UK.

Results

Enzymes involved in glycerol metabolism in Hansenula polymorpha

In order to elucidate the metabolic pathway for glycerol utilization in *Hansenula polymorpha*, a survey was made for the presence of various enzymes in extracts prepared from glycerol-grown cells. The enzymes glycerol oxidase and glycerol dehydratase were not detectable in these extracts and also no activity of glycerol kinase was found when using the assay described by Wieland and Suyter (1957). However, when these extracts were incubated with Mg^{2+}, ATP and glycerol, G3P formation was detected when using a discontinuous assay (assay 3 in the Methods section).

Glycerol kinase activity is generally determined using assay 1 described in the Methods section. This assay is performed at pH 9.8 in the presence of hydrazine in order to ensure efficient trapping of DHAP, one of the products of the reaction. While using assay 3, we observed that the optimum pH for the reaction catalyzed by glycerol kinase in *H. polymorpha* was between pH 7−8, although even at pH values of 5 and 10 significant activities remained (Fig. 1 A), and that addition of hydrazine hydrate caused strong inhibition (apparent K_i = 150 mM; Fig. 1 B). Using assay 3, specific activities of glycerol kinase in *H. polymorpha* wild type and strain 17B grown on glycerol in batch cultures

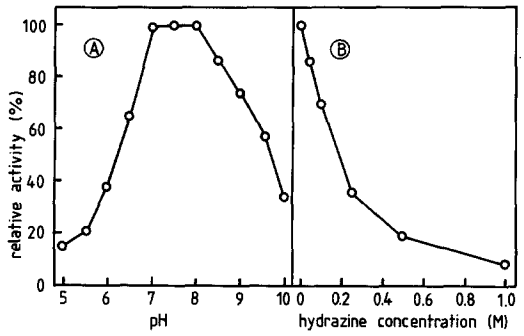

Fig. 1 A. Variation of glycerol kinase activity with pH. The specific activity of the enzyme was 250 nmol · min^{-1} · mg^{-1} of protein at pH 7.2. **B** Inhibition of glycerol kinase activity by hydrazine hydrate. The specific activity of the enzyme was 220 nmol · min^{-1} · mg^{-1} of protein in the absence of hydrazine. Cells were grown on 0.25% (w/v) glycerol. Assay 3 was used for measuring glycerol kinase activity (see Methods section)

Table 1. Profiles of enzymes related to glycerol metabolism during growth of wild type *Hansenula polymorpha* on various substrates. Cells were harvested in the mid-exponential growth phase. Activities are expressed as nmol · min^{-1} · mg^{-1} protein

Growth substrate	Enzyme				
	Glycerol kinase	G3P dehydrogenase [NAD(P)-independent]	DHA kinase[a]	DHA reductase[a]	DHAP reductase
Glucose	15	17	8	12	75
Ethanol	77	54	50	770	154
Xylose	317	54	110	755	139
Xylitol	209	46	125	2260	182
Ribose	246	31	95	1220	111
Sorbitol	194	77	95	150	106
Glycerol	250	50	120	980	82
DHA	124	39	150	675	67
Methanol	53	51	305	1290	113

[a] Data of de Koning et al. (1987)

were on average 250 and 315 nmol · min^{-1} · mg^{-1} of protein, respectively.

Two enzymes able to catalyze the conversion of G3P into DHAP were detected in *H. polymorpha* wild type grown on glycerol (Table 1). Activity of an NAD-dependent G3P dehydrogenase (DHAP reductase) was found when measured with NADH and DHAP as substrates. In addition, activity of NAD(P)-independent G3P dehydrogenase was observed in low spin (2,500 × *g*, 5 min) cell-free extracts. This PMS/MTT-dependent G3P dehydrogenase activity was completely sedimentable by centrifugation of crude extracts at 40,000 × *g* for 30 min. In cell fractionation experiments as described for *H. polymorpha* by Douma et al. (1985), the activity of this membrane-bound G3P dehydrogenase co-sedimented with the mitochondrial fraction (results not shown). Specific activities of the membrane-bound G3P dehydrogenase in *H. polymorpha* wild type and strain 17B grown on glycerol in batch cultures were on average 50 nmol · min^{-1} · mg^{-1} of protein.

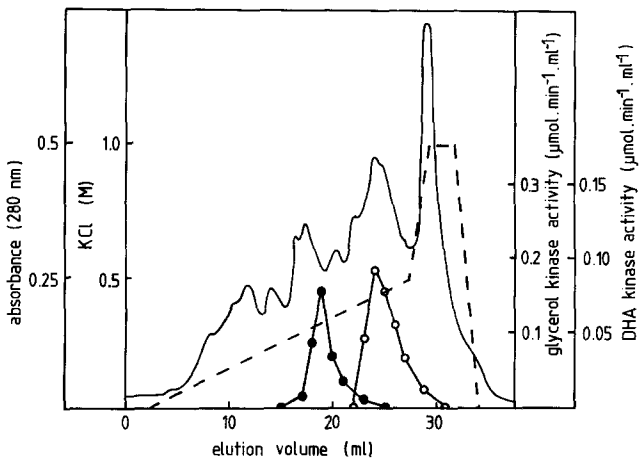

Fig. 2. DEAE-polyol HPLC elution profile of a cell-free extract of glycerol-grown (0.25%) cells of wild type *Hansenula polymorpha*. ——, absorbance at 280 nm; – – –, potassium chloride gradient; ●, dihydroxyacetone kinase; ○, glycerol kinase. Enzyme activities are expressed as µmol · min^{-1} · ml^{-1}

Separation of glycerol kinase and DHA kinase

When cell-free extracts of *H. polymorpha* wild type grown on glycerol were subjected to DEAE-polyol chromatography, the activities of glycerol kinase and DHA kinase could be separated (Fig. 2). During this purification step some inactivation of glycerol kinase occurred. The reason for this is not known and the enzyme was stable in cell-free extracts when kept on ice for several days. The partially purified glycerol kinase preparation thus obtained was used to determine some properties of the enzyme. For this purpose a continuous assay with pyruvate kinase/lactate dehydrogenase, which measures ADP formation (assay 2 in Methods section), was used. This assay worked properly with the partly purified enzyme, but not in crude cell-free extracts because of high endogenous activities most likely due to the presence of ATPases. The enzyme preparation exhibited Michaelis-Menten kinetics for glycerol and ATP with apparent K_m values of 1.0 and 0.5 mM, respectively. No activity was observed with DHA, glyceraldehyde, glycerate, erythritol, propanol, isopropanol, 1,2- and 1,3-propanediol and ethanediol, when tested at concentrations of 5 mM. The enzyme was not inhibited by 5 mM FBP.

Synthesis of enzymes related to glycerol metabolism during growth of Hansenula polymorpha on various carbon sources

To study the effect of the growth substrate on the synthesis of enzymes related to glycerol metabolism, *H. polymorpha* wild type was grown in batch culture on various carbon sources. The results (Table 1) show that both glycerol kinase and NAD(P)-independent G3P dehydrogenase remained repressed during growth with glucose and, in the case of glycerol kinase, also with ethanol and methanol. Following growth on glycerol, the activities of either enzyme was not significantly enhanced compared to the levels in cells grown on several other non-repressive substrates. NAD-dependent G3P dehydrogenase (DHAP reductase) was present at significant levels during growth on all substrates tested, indicating that the levels of this enzyme are little influenced by the carbon source used for growth. For a comparison

Table 2. Enzyme activities (nmol · min^{-1} · mg^{-1} protein) in cell-free extracts of *Hansenula polymorpha* strain 17B and glycerol-negative mutants derived from it. Cells were grown on sorbitol (0.25%, w/v) and harvested from the stationary growth phase

Strain	Enzyme				
	Glycerol kinase	G3P dehydrogenase [NAD(P)-independent]	DHA kinase	DHA reductase	DHAP reductase
17B	140	38	0	1700	395
17BG1	115	0	0	960	405
17BG3	100	0	0	1090	440
17BG6	78	0	0	2000	480
17BG51	0	25	0	3280	290
17BG71	0	33	0	2240	370
17BG81	0	40	0	1820	490

also the activities of DHA kinase and DHA reductase are included in Table 1 (see de Koning et al. 1987). Repression of the synthesis of these enzymes by glucose is clear, but compared to several other substrates glycerol does not cause a significant additional induction.

Isolation and characterization of mutants of Hansenula polymorpha strain 17B blocked in glycerol metabolism

In order to eliminate the possibility that the glycerol phosphorylative pathway might be bypassed via DHA, glycerol-negative mutants of *H. polymorpha* were selected in strain 17B (de Koning et al. 1987). Following EMS treatment and nystatin enrichment with glycerol as the non-permissive substrate, several independent glycerol-negative mutants were isolated. Enzyme analysis of sorbitol-grown cells revealed that three of these mutants (strains 17BG1, 17BG3, 17BG6) were blocked in the NAD(P)-independent G3P dehydrogenase and three (strains 17BG51, 17BG71, 17BG81) in glycerol kinase (Table 2). Strain 17BG6 was crossed to an appropriate wild type strain carrying two marker mutations (adenine plus methionine auxotrophy) and diploids selected able to grow on methanol. The diploids were sporulated and the haploid segregants again tested for growth on glycerol and DHA. As shown in Table 3, four different classes of mutants were obtained. An analysis of enzymes synthesized by representatives of the various classes revealed that NAD(P)-independent G3P dehydrogenase was absent in class 2 mutants, DHA kinase in class 3 mutants and DHA kinase plus NAD(P)-independent G3P dehydrogenase in class 4 mutants. Further investigations showed that, compared to strain 17B or wild type, class 2 mutants were only able to grow very slowly in glycerol mineral media. The substrate concentration in the medium was found to influence the growth rate of strain 17BG6.10 and doubling times of 26 h and 15 h were observed with 0.25% and 5% glycerol (w/v), respectively. These observations are taken to indicate that in wild type *H. polymorpha* glycerol is metabolized mainly via the phosphorylative pathway.

Glycerol metabolism in various yeasts

A survey was made of the presence of enzymes related to glycerol metabolism in various methylotrophic and non-methylotrophic yeast species, grown on glycerol (Table 4). *Schizosaccharomyces pombe* CBS 356 and *Candida valida* CBS 5759 were initially included in this study, but these strains failed to grow on glycerol. Glycerol kinase activities were detected in all glycerol-positive yeasts tested when the discontinuous assay for G3P formation was used (assay 3 in the Methods section). Only with *Kluyveromyces marxianus* the activity of the enzyme was clearly too low to explain the growth rate observed with glycerol. Also the second enzyme of the phosphorylative pathway, NAD(P)-independent G3P dehydrogenase was detected in low spin extracts of all organisms. Since NAD-dependent G3P dehydrogenase (DHAP reductase) activity also was observed, the possibility cannot be excluded that, at least in some of these organisms, this enzyme plays a role in glycerol catabolism. Whereas DHA reductase activity was exceptionally high in *H. polymorpha*, DHA kinase and glycerol kinase were found to be present at high levels in *Pichia pastoris* and *Pichia anomala*. The activity of the DHA kinase in *H. polymorpha* generally is measured with 0.25 mM DHA in order to minimize DHA reductase activity also present in the extract (de Koning et al. 1987). With *P. pastoris* and *P. anomala*, however, maximum activities of DHA kinase were only obtained with 40 mM of DHA. Since glycerol kinase in general also employs DHA as a substrate (Thorner and Paulus 1973), the kinetics of the DHA kinase activity in *P. pastoris* was studied in more detail. The reaction velocity as a function of the DHA concentration was not hyperbolic: double reciprocal plots displayed a downward curvature (Fig. 3), indicating two apparent K_m values for DHA of 0.7 and 10 mM. When assaying for DHA kinase activity with 1 mM of DHA, glycerol at a concentration of 5 mM inhibited the rate of DHAP formation for 75%. In methanol-grown cells of *P. pastoris* DHA kinase was present at an activity of 550 nmol · min^{-1} · mg^{-1} protein. This enzyme possessed a very high affinity for DHA (apparent K_m value approximately 3 μM) and was not inhibited by the presence of glycerol.

Discussion

Following the isolation of a DHA kinase-negative mutant of *Hansenula polymorpha* (de Koning et al. 1987) unimpaired in its ability to grow on glycerol, we decided to reinvestigate glycerol metabolism in methylotrophic yeasts. Only when using assay conditions (assay 3) that resemble the intracellular situation more closely (with respect to pH and concentrations of substrates), glycerol kinase activities were detected in *H. polymorpha*, sufficiently high to explain the observed growth rate (0.42 h^{-1}). The properties of the enzyme in *H. polymorpha* are different from most other glycerol kinases described (Thorner and Paulus 1973; Thorner 1975) in that it has a pH optimum around 7.5 (compared with 9–9.8 in other organisms) and, most importantly, is strongly inhibited by hydrazine hydrate. These properties render the assay of Wieland and Suyter (1957), which is generally used for glycerol kinase, unsuitable. The enzyme detected in *H. polymorpha* is unusual in a number of other species as well. The apparent K_m values for glycerol and ATP are relatively high and the enzyme is very specific for glycerol, whereas

Table 3. Phenotypes and enzyme activities (nmol · min^{-1} · mg^{-1} of protein) in cell-free extracts of representatives of the four classes of segregants obtained from crossing *H. polymorpha* strain 17BG6 and wild type cells. Cultures were grown on sorbitol (0.25%, w/v) and harvested from the stationary growth phase

Class	Strain	Ability to grow on		Enzyme				
		DHA	Glycerol	Glycerol kinase	G3P dehydrogenase	DHA kinase	DHA reductase	DHAP reductase
1	17BG6.17	+	+	310	57	136	2330	470
2	17BG6.10	+	±	115	0	110	1360	560
3	17BG6.3	−	+	200	45	0	2560	740
4	17BG6.20	−	−	90	0	0	2320	760

Table 4. Doubling times (h) and profiles of enzymes related to glycerol metabolism during growth of various yeast species with glycerol (0.25%, w/v). Cells were harvested in the mid-exponential growth phase. Enzyme activities are expressed as nmol · min^{-1} · mg^{-1} protein

Organism	Doubling time (h)	Enzyme				
		Glycerol kinase	G3P dehydrogenase	DHA kinase	DHA reductase	DHAP reductase
Hansenula polymorpha	1.6	250	50	120	980	82
Pichia pastoris	2.3	1330	89	953	22	121
Pichia pinus	7.0	121	24	43	18	121
Pichia anomala	1.5	1050	99	1390	0	24
Candida boidinii	4.0	117	12	25	173	51
Candida methylica	6.0	91	6	36	318	111
Rhodotorula graminis	24	14	90	17	8	13
Kluyveromyces marxianus	2.0	14	46	0	225	102
Wickerhamiella domercqii	3.5	97	12	0	11	54

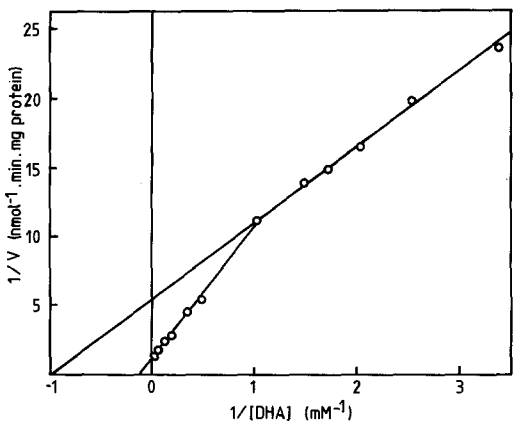

Fig. 3. Double reciprocal plot of the rate of DHA phosphorylation as a function of DHA concentration in cell-free extract of glycerol-grown (0.25%) cells of *Pichia pastoris*

most other glycerol kinases also show activity with DHA and glyceraldehyde (Thorner and Paulus 1973). The *H. polymorpha* enzyme is not inhibited by FBP, as are most bacterial glycerol kinases, and in this aspect it resembles the enzyme of *Candida mycoderma* (Grunnet and Lundquist 1967).

The G3P produced by the glycerol kinase is further metabolized via a membrane-bound G3P dehydrogenase. As far as investigated, the enzyme in *H. polymorpha* does not differ significantly from enzymes found in other organisms. The activity measured in cell-free extracts of glycerol-grown cells is too low (by a factor 5) to explain the observed growth rate. The reason for this might be that for maximum activity of the enzyme the mitochondria need to be intact. This was indicated by the fact that the enzyme displayed a 50% higher activity in osmotically disrupted protoplasts (data not shown).

The essential role played by both glycerol kinase and membrane-bound G3P dehydrogenase in glycerol metabolism became evident by the isolation and characterization of glycerol-negative mutants of *H. polymorpha* strain 17B (DHA kinase-negative) blocked in these enzymes (Table 2). Among the progeny of a mating of strain 17BG6 with wild type, strains were identified which were still G3P dehydrogenase-negative but had regained DHA kinase (class 2; Table 3). Growth of these strains on DHA was normal but only very slow growth on glycerol was observed. It thus appears that under in vivo conditions glycerol is only metabolized very slowly via the oxidative pathway, although the enzymes glycerol dehydrogenase (= DHA reductase) and DHA kinase are present in significant levels. Relatively slow growth on glycerol has been reported previously for organisms which normally metabolize glycerol via DHA (e.g. *Schizosaccharomyces pombe*; May et al. 1982) or do so because of a mutational inactivation of glycerol kinase (e.g. *E. coli*, Martin et al. 1977; *Cellulomonas* sp. NT 3060, Nishise et al. 1985). The reverse reaction from DHA to glycerol also was found to proceed very slowly in a DHA kinase-negative mutant of *H. polymorpha*, strain 17B, growing on media with high DHA concentrations (de Koning et al. 1987). The reason for these low in vivo rates is not known.

The failure of double mutants lacking DHA kinase and NAD(P)-independent G3P dehydrogenase activities to grow

on glycerol also showed that the NAD-dependent G3P dehydrogenase does not function in glycerol metabolism. Possible reasons are that the equilibrium of the reaction strongly favours G3P formation or that the enzyme is localized in peroxisomes (Kawamoto et al. 1979) where it may serve in a shuttle mechanism for reducing power. The observed increase in activity in stationary cells might then be due to the utilization of fat the metabolism of which is partly localized in the peroxisomes (Kawamoto et al. 1979). Egli and Lindley (1984), however, obtained no evidence for the operation of a G3P shuttle in *Candida boidinii*, grown on mixtures of methanol and glucose. They reported that in this organism the NAD-dependent G3P dehydrogenase was absent and did not test for the equivalent membrane-bound enzyme.

Synthesis of both glycerol kinase and G3P dehydrogenase appears to be regulated by a repression/derepression mechanism and no clear induction during growth on glycerol was found (Table 1). A possible role for glycerol as an osmoticum to counteract low water activities (high salt or sugar concentrations), as has been shown for the yeast *Debaryomyces hansenii* (Adler et al. 1985) and for several fungi (Hocking 1986), may explain the absence of an induction mechanism.

Since glycerol metabolism in *H. polymorpha* turned out to be different from what was reported in the literature, other yeasts were screened for the presence of the phosphorylative pathway. With the exception of *Kluyveromyces marxianus*, sufficiently high levels of glycerol kinase were found in all yeast species tested to explain the observed growth rates on glycerol (Table 4). This was also the case with five yeast species (*H. polymorpha*, *Pichia pini*, *C. boidinii*, *Candida methylica* and *Wickerhamiella* [= *Torulopsis*] *domercqii*) previously reported (Babel and Hofmann 1982) to lack these enzymes. The activities of DHA reductase in these strains were found to be much lower than reported by Babel and Hofmann (1982). However, a different assay was used, and with *H. polymorpha* we observed that DHA reductase levels may strongly fluctuate, dependent on the phase of growth on sorbitol (Tables 1 and 2, midexponential and stationary phase, respectively). In *Pichia pastoris* and *P. anomala* glycerol kinase activities are much higher than necessary to explain the growth rate. The kinetics of the DHA kinase activity in *P. pastoris* (Fig. 3), and its inhibition by glycerol (in glycerol-grown cells but not in methanol grown cells), suggest that at least in this organism glycerol kinase also catalyzes the conversion of DHA into DHAP.

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